Induction of alternative fate other than default neuronal fate of embryonic stem cells in a membrane-based two-chambered microbioreactor by cell-secreted BMP4

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Cell-secreted soluble factor signaling in a diffusion dominant microenvironment plays an important role on early stage differentiation of pluripotent stem cells *in vivo*. In this study, we utilized a membrane-based two-chambered microbioreactor (MB) to differentiate mouse embryonic stem cells (mESCs) in a diffusion dominant microenvironment of the top chamber while providing enough nutrient through the bottom chamber. Speculating that accumulated FGF4 in the small top chamber will augment neuronal differentiation in the MB culture, we first differentiated mESCs for 8 days by using a chemically optimized culture medium for neuronal induction. However, comparison of cellular morphology and expression of neuronal markers in the MB with that in the 6-well plate (6WP) indicated relatively lower neuronal differentiation in the MB culture. Therefore, to investigate whether microenvironment in the MB facilitates non-neuronal differentiation, we differentiated mESCs for 8 days by using chemically defined basal medium. In this case, differentiated cell morphology differed markedly between the MB and 6WP cultures: epithelial sheet-like morphology in the MB, whereas rosette morphology in the 6WP. Expression of markers from the three germ layers indicated lower neuronal but higher meso- and endo-dermal differentiation of mESCs in the MB than the 6WP culture. Moreover, among various cell-secreted soluble factors, BMP4 expression was remarkably upregulated in the MB culture. Inhibition of BMP4 signaling demonstrated that enhanced effect of upregulated BMP4 was responsible for the prominent meso- and endo-dermal differentiation in the MB. However, in the 6WP, downregulated BMP4 had a minimal influence on the differentiation behavior. Our study demonstrated utilization of a microbioreactor to modulate the effect of cell-secreted soluble factors by autoregulation and thereby inducing alternative self-capability of mESCs. Understanding and implementation of autoregulation of soluble factors similar to this study will lead to the development of robust culture systems to control ESC behavior. © *2012 American Institute of Physics*. [http://dx.doi.org/10.1063/1.3693590]

I. INTRODUCTION

Embryonic stem cells (ESCs) preserve their pluripotency as undifferentiated cells for long-term *in vitro*. Upon their differentiation, they can give rise all kind of cells of an animal body. These capabilities of ESCs make them an unlimited source for deriving various mature cells such as hepatocytes, cardiomyocytes, etc. Therefore, ESCs hold great promises in regenerative medicine and drug industry. However, to realize the promises, it is necessary to direct ESCs towards a specific cell type by controlling their usual random differentiation. Understanding

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ESC microenvironment and implementing the gained knowledge will enable better control over the differentiation process.[1]

Soluble factors in the microenvironment play an important role on ESC undifferentiation and differentiation behaviors. Exogenous soluble factors can be easily added to cultures to influence ESC behavior. However, ESCs are very sensitive to the autocrine/paracrine signaling induced by cell-secreted factors.[2,3] Moreover, ESCs can undergo early stage differentiation spontaneously indicating a strong self-autonomous behavior of ESCs modulated by cell-secreted factors as well as other microenvironmental clues.[4] Therefore, for establishing an *in vitro* differentiation system, it is essential to consider influence of exogenously added soluble factors as well as cell-secreted ones (i.e., endogenous soluble factors) on cell behavior.[5]

A cell-secreted soluble factor in a conventional open macro-scale static culture system, e.g., 6-well plate (6WP), becomes diluted in a large volume compared to cellular volume. Moreover, inherent Marangoni convection (caused by surface tension differences in the free liquid-air interface) in these culture systems rapidly sweeps the soluble factors away from the cell neighborhood.[6] However, in enclosed micro-scale static culture systems, cellular microenvironment is more diffusion dominant than the macro-scale owing to the small dimension as well as the absence of free liquid-air interface.[6] Therefore, in these culture systems, cell-secreted soluble factors can accumulate in a small volume as well as remain in the cell neighborhood for long-time because of the diffusion dominant nature of the microenvironment. These capabilities of the micro-scale culture systems provide opportunity to study influence of cell-secreted soluble factors on cell behavior in an *in vivo* relevant dimension and manner. However, previous microfabrication based studies primarily focused on flow[7,8] and size (diameter of ESC aggregates i.e., embryoid bodies (EBs))[9,10] dependent modulation of soluble microenvironment to direct ESC differentiation. No study focused on the influence of cell-secreted factors in a small culture volume, which might strongly influence ESC differentiation. Characterization of this influence is important for developing robust culture systems to control ESC differentiation through a better understanding of soluble factor signaling.

In this context, we were interested to study ESC behavior in an *in vivo* mimicking microenvironment, which can realize accumulation and retention of cell-secreted factors around the cells. In fact, pluripotent stem cells are enclosed by trophectoderm and extra-embryonic part *in vivo*. Early fate change of the stem cells occurs in a diffusion dominant microenvironment of the enclosure while nutrient supply to the enclosed cells is provided by maternal side. Therefore, to culture and differentiate mouse embryonic stem cells (mESCs) in an *in vivo* mimicking microenvironment, we are interested in utilizing a membrane-based two-chambered microbioreactor (MB).

In a previous study, to investigate how cell-secreted factors influence mESC pluripotency by auto-regulation, we cultured mESCs in the MB (Fig. 1) by using chemically defined medium (CDM) containing LIF (leukemia inhibitory factor).[11] During the culture, we maintained small culture volume in the top chamber to form the diffusion dominant microenvironment, while culture volume in the bottom chamber was adjusted to provide enough nutrient supply for static culture (Fig. 1). We observed that pluripotent culture of mESCs in the MB maintained higher pluripotency than conventional macro-scale cultures (e.g., 6WP) owing to an enhanced effect of up-regulated cell-secreted BMP4.[11] This result suggested that actions of various cell-secreted factors might also have an enhanced effect on mESC fate choice in the MB. Understanding of the effect is important to establish an adequate signaling environment to enhance autonomous behavior of EB in a microbioreactor (e.g., MB) or robust control on ESC behavior in microcapsule. Consequently, in this study, we investigated how cell-secreted soluble factors modulate early stage differentiation of mESCs in the MB as compared with macro-scale 6WP culture by using CDM without LIF.

II. MATERIALS AND METHODS

A. Design and fabrication of the MB

Design detail and fabrication of the MB is described elsewhere.[11] In summary, a porous polyester membrane (pore size 0.4 μm; Corning Inc.) was sandwiched between the two

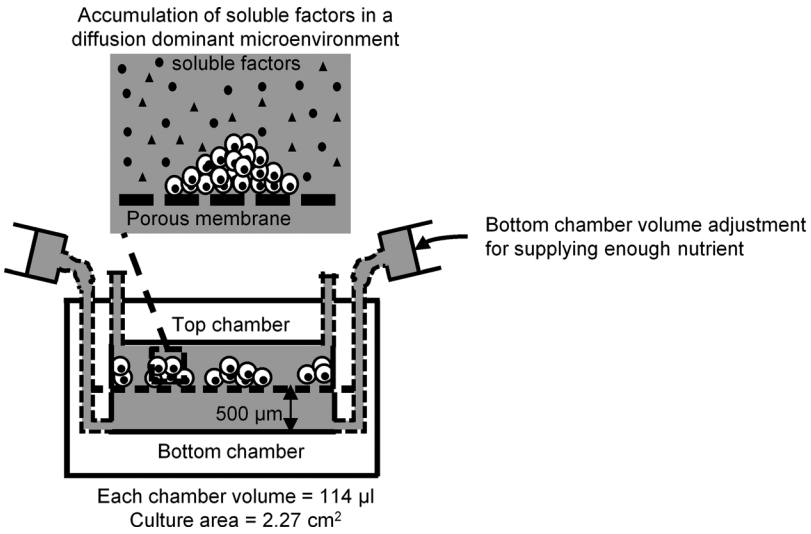

FIG. 1. Schematic showing cross section of the membrane-based two-chambered microbioreactor (MB). Dashed lines represent silicone tubing (ID = 1 mm) connected to feeding holes of the top and bottom chambers. mESCs are inoculated on top face of membrane; differentiated in diffusion dominant microenvironment formed in the small top chamber while providing enough nutrients through the bottom chamber. Culture medium volume in syringes connected to the bottom chamber tubing can be adjusted to maintain equal culture volume to surface area ratio between the MB and 6WP. In case of the top chamber syringes, there is no excess culture medium volume (for details, see Sec. II C).

chambers (diameter 17.25 mm, height 500 μm, and volume 114 μl) which were made from polydimethylsiloxane (PDMS) polymer by using common microfabrication methods. Each chamber had two feeding holes to exchange culture medium independently (Fig. 1). Cells were cultured on the top face of the membrane, and culture area was 2.27 cm^2.

B. Routine cell culture

Routine culture of mESCs was performed as described in our previous work.[11] Briefly, mESCs were inoculated at 4×10^5 cells/60-mm gelatin-coated dish (Iwaki) and cultured with a culture medium containing 20% ESC qualified-FBS (Gibco) in high-glucose DMEM (Gibco), 1000 U/ml ESGRO-LIF (Chemicon), and other basic constituents. Cells were subcultured every other day. Cells were maintained in a 37 °C humidified environment containing 20% O_2 and 5% CO_2.

C. Neuronal differentiation in the 6WP and MB by using RHB-A medium

RHB-A is a chemically optimized medium for efficient differentiation of mESCs towards neuronal lineage (Stem cell sciences, UK). Therefore, this medium was used for efficient neuronal differentiation in the MB and 6WP. Before inoculation of cells, MB was sterilized by ethanol, coated with gelatin by applying 0.1% (w/v) gelatin solution, and pre-incubated several hours with RHB-A medium. mESCs were inoculated on the membrane of the MB as well as in the gelatin coated 6WP at 1×10^4 cells/cm^2. Cells were differentiated for 8 days in a 37 °C humidified environment containing 5% O_2 and 5% CO_2.

After inoculation, culture medium volume of the MB was adjusted to maintain equal ratio of volume to the surface area (0.2 ml/cm^2) between the MB and 6WP (diameter 34.6 mm, surface area 9.4 cm^2, and culture medium volume 2 ml) cultures. For the adjustment, syringes containing 0.1 ml culture medium were connected with tubing of the bottom chamber feeding holes (Fig. 1). However, syringes connected with the top chamber feeding holes did not have any excess culture medium volume, and culture volume in the top chamber was maintained at 114 μl (Fig. 1). This approach allowed accumulation of soluble factors in the small volume of the top chamber, while large volume of culture medium in the bottom chamber provided

enough nutrient supply for static culture in the MB. Culture medium of the both chambers of MB and the 6WP were changed every other day.

D. Differentiation in the 6WP and MB by using chemically defined basal medium (CD-BM)

Pre-treatment of the MB was done as described before. mESCs were inoculated on the membrane of MB as well as in the gelatin coated 6WP at 1×10^4 cells/cm^2. Cells were differentiated for 8 days by using chemically defined basal medium (CD-BM) which composition was: 15% knockout serum (KSR; Gibco) in knockout DMEM (Gibco), 1% MEM non-essential amino acids (Gibco), 2 mM GlutaMax-I (Gibco), and 0.1 mM 2-mercaptoethanol (Gibco). KSR was used because it contains fewer extrinsic proteins. Cells were differentiated for 8 days in a 37 °C humidified environment containing 5% O_2 and 5% CO_2. Culture medium volume adjustment and exchanging protocol was done as described before.

E. Soluble factor signaling inhibition experiment

Inhibition experiments were performed in differentiation culture by using CD-BM. For these experiments, BMP4 antagonist Noggin[12] (R&D Systems) at 100 ng/ml was added to the culture medium.

F. qPCR analysis

Isolation of total mRNA from the culture systems on day 8, cDNA synthesis, and qPCR were performed as described in our previous study.[11] Primers for cDNA amplification are listed in Table I. Primer sequences for Rex1, Foxa2, FGF4, and BMP4 were designed by PerlPrimer[13] and others except Rpl13a were taken from PrimerBank database.[14] Primer sequences for Rpl13a were kindly provided by Kevin Montagne, For each gene, qPCR reaction for each sample was performed in duplicate. Raw data of PCR product amplification curves were analyzed using LINREGPCR V11.4 software[15] to determine the threshold cycles (C_t). A BestKeeper index[16] for each sample was calculated by geometric mean of C_t's of reference genes: Rpl13a, Ppia, and Hprt1. A gene expression level in a sample relative to the respective expression in mESC culture (cultured for 2 days) was calculated by the following:

$$\text{Relative expression level} = \frac{2^{C_t(\text{Bestkeeper})_{\text{sample}} - C_t(\text{target gene})_{\text{sample}}}}{2^{C_t(\text{Bestkeeper})_{\text{mESCs}} - C_t(\text{target gene})_{\text{mESCs}}}}.$$

G. Statistical analysis

Student's *t*-test was performed for statistical evaluation using demo version of GRAPHPAD software (GraphPad Software, Inc.). Difference with a $P < 0.05$ was considered to be statistically significant. All data are presented as the mean ± standard error of the mean (SEM).

III. RESULT AND DISCUSSION

A. Neuronal differentiation in the 6WP and MB by using RHB-A culture medium

mESCs secret FGF4 extensively;[17] its signaling induces mESCs to exit pluripotency.[18] In chemically defined medium, autocrine FGF signaling induces prominent neuronal differentiation in macro-scale (e.g., 6WP) differentiation culture.[19–22] Therefore, we speculated that enhanced FGF signaling owing to accumulation of FGF4 in the small top chamber of the MB will further augment the neuronal differentiation. To validate the speculation, we compared neuronal differentiation of mESCs between the MB and 6WP cultures. For the differentiation, we used RHB-A culture medium which is very efficient for inducing neuronal differentiation[23] and contains no FGFs (stem cell sciences, UK).

TABLE I. Genes and primers used in qPCR analysis.

Description	Genes	Primers	Sequences (5' – 3')
Reference gene	Rpl13a	Forward	TCTGGAGGAGAAACGGAAGGA
		Reverse	GGTTCACACCAAGAGTCCATTG
	Hprt1	Forward	TCAGTCAACGGGGGACATAAA
		Reverse	GGGGCTGTACTGCTTAACCAG
	Ppia	Forward	GAGCTGTTTGCAGACAAAGTT
		Reverse	CCCTGGCACATGAATCCTGG
Pluripotency marker	Rex1	Forward	ACACAGAAGAAAGCAGGAT
		Reverse	GAACAATGCCTATGACTCAC
	Dppa3	Forward	GACCCAATGAAGGACCCTGAA
		Reverse	GCTTGACACCGGGGTTTAG
Neuronal marker	Nestin	Forward	CTACATACAGGACTCTGCT
		Reverse	GTCTCAAGGGTATTAGGCA
	Map2	Forward	GCCAGCCTCAGAACAAACAG
		Reverse	AAGGTCTTGGGAGGGAAGAAC
	Tubb3 (Tubulin, beta 3)	Forward	TAGACCCCAGCGGCAACTAT
		Reverse	GTTCCAGGTTCCAAGTCCACC
Mesoderm marker	Nkx2.5	Forward	GACAAAGCCGAGACGGATGG
		Reverse	CTGTCGCTTGCACTTGTAGC
	Flk1	Forward	TTTGGCAAATACAACCCTTCAGA
		Reverse	GCAGAAGATACTGTCACCACC
	Pdgfrb	Forward	CAACTCACTAGGGCCGGAG
		Reverse	GCACGGAATTGTCGTCTCAG
Endoderm marker	Sox17	Forward	ACCTACACTTACGCTCCAGTC
		Reverse	GCCGTAGTACAGGTGCAGAG
	Foxa2	Forward	GCCAGCGAGTTAAAGTATG
		Reverse	CATGCTCATGTATGTGTTCA
	Ttr	Forward	ATCGTACTGGAAGACACTTGGC
		Reverse	CCGTGGTGCTGTAGGAGTAT
Cell-secreted soluble factor	FGF4	Forward	TCGGTGTGCCTTTCTTTACC
		Reverse	ACCTTCATGGTAGGCGACAC
	BMP4	Forward	CCATCACGAAGAACATCTG
		Reverse	AATGTTTATACGGTGGAAGC
	Nodal	Forward	TTCAAGCCTGTTGGGCTCTAC
		Reverse	TCCGGTCACGTCCACATCTT
	Activin	Forward	ATAGAGGACGACATTGGCAGG
		Reverse	CACGCTCCACTACTGACAGG

When we compared cellular morphology and expression of neuronal markers between the MB and 6WP cultures, we did not observe the speculated enhanced effect of FGF4 in the MB over 6WP culture. Although differentiated cells at day 8 formed typical rosette morphology indicating neuronal differentiation in both cultures, neuronal extension—a common indicator of the degree of neuronal differentiation—was less extensive in the MB than the 6WP culture (Fig. 2). Gene expression analysis showed that expression of two neuronal markers (Nestin and Map2) among three (other one is Tubb3) was significantly lower in the MB than the 6WP culture (Fig. 3(b)). Therefore, morphology as well as expression of neuronal markers showed relatively lower neuronal differentiation in the MB. In addition, higher expression of pluripotency marker Dppa3 in the MB indicated overall differentiation in the MB was lower than the 6WP culture (Fig. 3(a)). These results nullified our initial speculation: accumulation of FGF4 in the small top chamber of the MB will augment the neuronal differentiation.

B. Differentiation by using CD-BM

Inhibition of neuronal differentiation even in the RHB-A culture medium indicated: (1) non-neuronal differentiation may be favored in the MB and (2) other soluble factor than FGF4

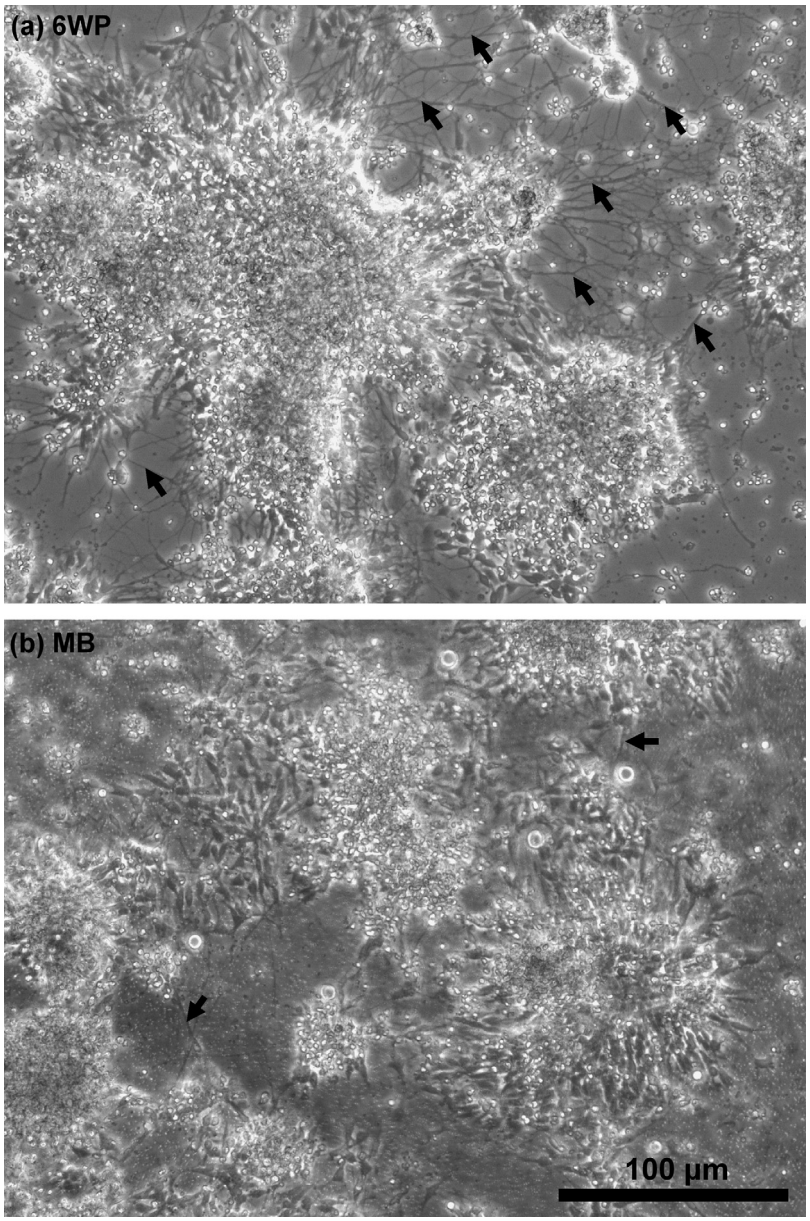

FIG. 2. Differentiated cells in the 6WP (a) and MB (b) cultures at day 8 by using RHB-A medium, an optimum medium for neuronal differentiation. Neuronal extensions (e.g., some are marked by arrows) are less frequently observed in the MB than the 6WP culture. Scale bar represents 100 μm.

might have controlled the mESC differentiation in the MB. To investigate these, we differentiated mESCs in the MB by using CD-BM and characterized: the overall differentiation behavior of the cultures by analyzing neuronal as well as meso- and endo-dermal markers and effects of soluble factors.

After 8 days of differentiation, there was significant difference in the cellular morphology between the 6WP and MB cultures. In the 6WP culture, widespread neuronal differentiation was evident by the presence of extensive neuronal extensions (Fig. 4(a))—almost similar to the observation in the respective differentiation culture by using RHB-A medium (Fig. 2(a)). On the contrary, complete reversal of the rosette morphology observed in the RHB-A medium (Fig. 2(b)) to epithelial sheet-like morphology in the CD-BM (Fig. 4(b)) showed prominent non-neuronal differentiation in the MB.

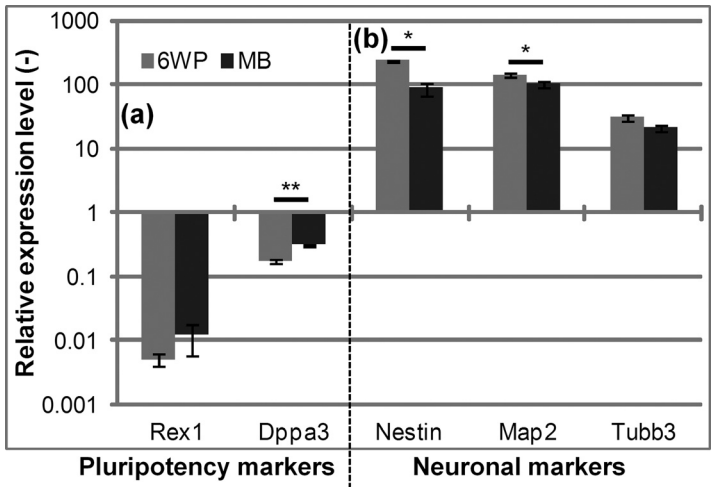

FIG. 3. Relative gene expression levels (in Log_{10}-scale) in the MB and 6WP differentiation cultures at day 8 by using RHB-A medium, an optimum medium for neuronal differentiation. Expression level one represents gene expression in the undifferentiated mESCs cultured for 2-days. Columns and error bars represent mean ± SEM of three cultures. Statistical significances are shown using symbols * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

Gene expression of cell populations in the 6WP and MB cultures correlated well with the respective morphological observations and revealed suppression of neuronal and enhancement of meso- and endo-dermal differentiation in the MB culture. In contrast to the relatively upregulated expression of pluripotency markers in the MB culture by using RHB-A medium (Fig. 3(a)), expression of pluripotency markers (Rex1 and Dppa3) decreased equally in both cultures upon differentiation in the CD-BM medium (Fig. 5(a)). In accordance with the morphological difference (Fig. 4), expression of two neuronal markers (Map2 and Tubb3) among three (other is Nestin) was significantly downregulated in the MB than the 6WP culture (Fig. 5(b)). Instead, expressions of meso- and endo-dermal markers were upregulated in the MB than the 6WP culture. All three mesodermal markers (Nkx2.5, Flk1, and Pdgfrb) and two endodermal markers (Sox17 and Foxa2) among three (other is Ttr) were significantly upregulated in the MB (Figs. 5(c) and 5(d)). We, therefore, concluded that microenvironment in the MB was supportive to meso- and endo-dermal differentiation but inhibitive to neuronal differentiation.

To identify which soluble factor prominently controlled the microenvironment of the MB, we analyzed expression of various soluble factors: FGF4, BMP4, Nodal, and Activin. We selected these soluble factors on the basis of their influence on mESC differentiation: autocrine FGF4 induces neuronal differentiation; exogenous BMP4 inhibits neuronal but induces meso- and endo-dermal differentiation; and Nodal or Activin induces meso- and endo-dermal differentiation.[24] Among these, only BMP4 expression was remarkably upregulated in the MB as compared to the 6WP culture (Fig. 5(e)).

C. Inhibition of BMP4 signaling in the differentiation cultures by using CD-BM

Various previous studies showed that exogenous BMP4 suppresses neuronal and enhances non-neuronal fate choices.[19,25,26] Therefore, we speculated that cell-secreted upregulated BMP4 in the MB also influenced the differentiation in a similar fashion. To confirm this, we inhibited BMP4 signaling in the differentiation cultures by using CD-BM.

We inhibited BMP4 signaling by its antagonist Noggin. Morphological change as well as change in gene expression by the inhibition confirmed effect of BMP4 in the MB culture. Inhibition of BMP4 signaling caused no apparent morphological change in the 6WP culture (Figs. 6(a) and 6(b)). However, epithelial sheet-like morphology became loose and scattered in the MB by the inhibition (Figs. 6(c) and 6(d)). Comparison of gene expression between the inhibited and non-inhibited cultures agreed well with the morphological observations and confirmed the prominent role of BMP4 signaling only in the MB. In the 6WP, BMP4 inhibition did not

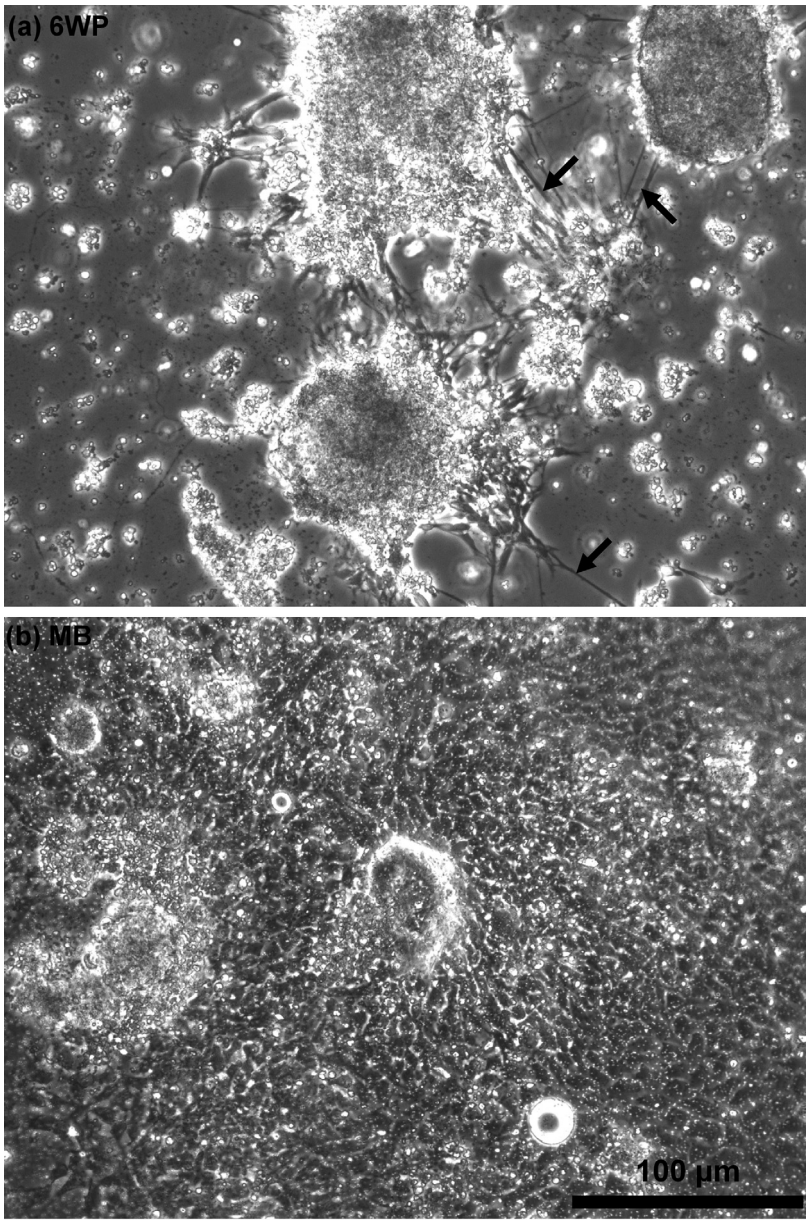

FIG. 4. Morphology of the differentiated cells in the 6WP (a) and MB (b) at day 8 by using CD-BM. Differentiated cells in the 6WP show extensive neuronal extensions (e.g., some are marked by arrows) but cells in the MB form epithelial sheet-like morphology. Scale bar represents 100 μm.

cause any change in the expression of the pluripotency markers as well as germ layers markers (Fig. 7(a)). Similar to the 6WP, expression of neuronal markers were unchanged by BMP4 inhibition in the MB (Fig. 7(b)). However, pluripotency marker (Rex1) was upregulated whereas mesodermal (Nkx2.5 and Pdgfrb) and endodermal (Sox17 and Ttr) markers were downregulated significantly by BMP4 inhibition in the MB (Fig. 7(b)). Therefore, we concluded that cell-secreted BMP4 superseded the effect of FGF signaling and induced meso- and endo-dermal differentiation in the MB culture (Figs. 5 and 7(b)). However, in the 6WP culture, cell-secreted BMP4 signaling was negligible (Fig. 7(a)), and mESC differentiation was directed towards neuronal lineage (Fig. 5) by FGF signaling as usual.[19–22] These results also suggested that enhanced effect of cell-secreted BMP4 in the MB presumably inhibited the neuronal differentiation in RHB-A medium (Figs. 2 and 3).

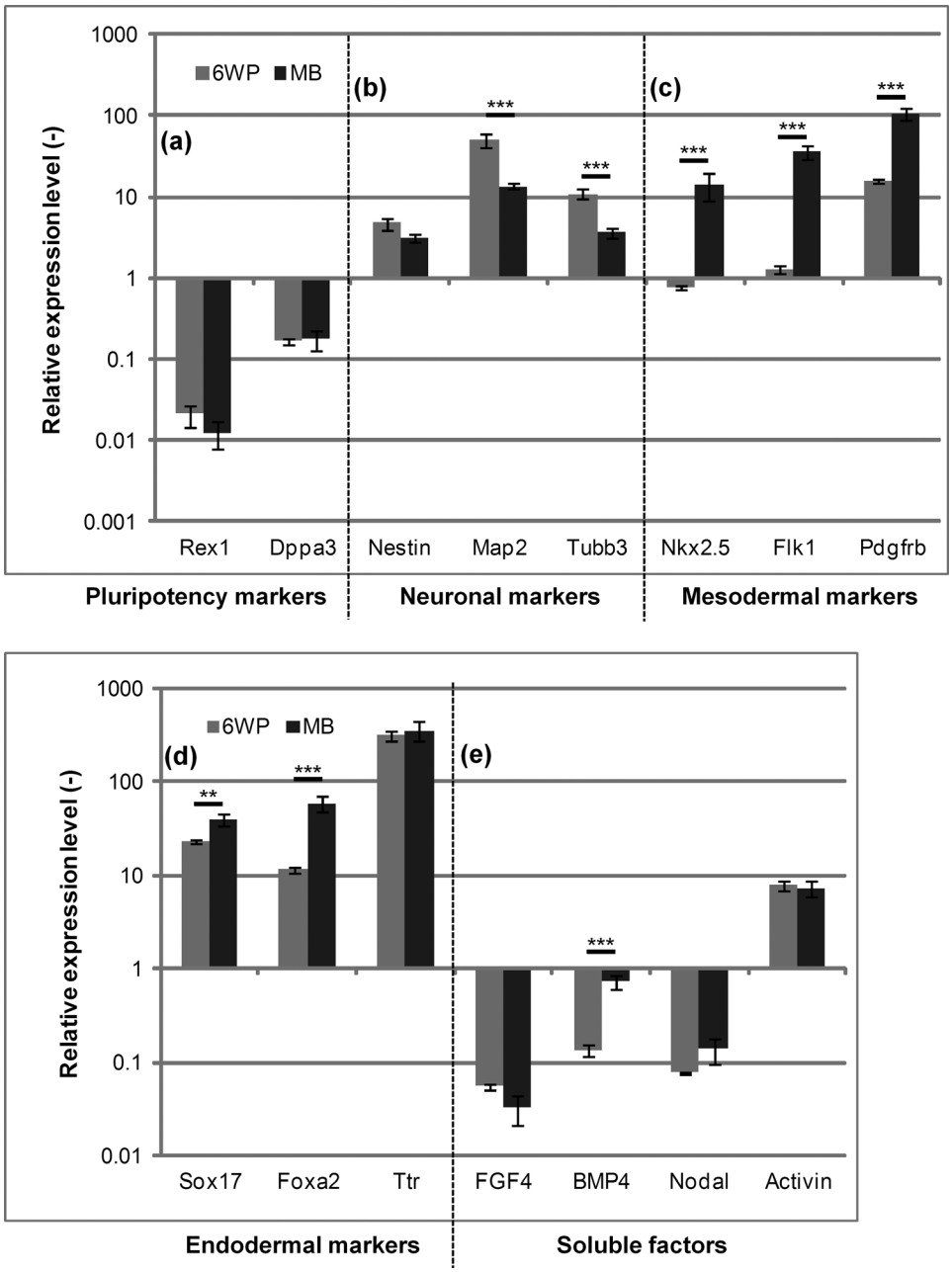

FIG. 5. Relative gene expression levels (in Log_{10}-scale) of pluripotency markers (a); lineage specific differentiation markers ((b), (c), and (d)); and soluble factors (e) in the 6WP and MB cultures after 8-days differentiation of mESCs by using CD-BM. Expression level one represents gene expression in the undifferentiated mESCs cultured for 2-days. Columns and error bars represent mean ± SEM of five to six cultures. Statistical significances are shown using symbols * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

Only in the micro-scale MB culture, we realized an auto-regulatory loop—to modulate mESC pluripotency[11] and differentiation (in this study) by cell-secreted BMP4. In pluripotent culture of mESCs, exogenous BMP4 co-operates LIF to maintain mESC pluripotency by resisting mESC differentiation which is activated by autocrine FGF4 signaling.[2] In this scenario, BMP4 blocks neuronal differentiation, which is otherwise partially prevented by LIF; on the other hand, LIF blocks meso- and endo-derm differentiation induced by BMP4.[19] In the absence of LIF i.e., differentiation culture, exogenous BMP4 activates meso- and endo-dermal differentiation as well as inhibit neuronal differentiation which is activated by autocrine FGF

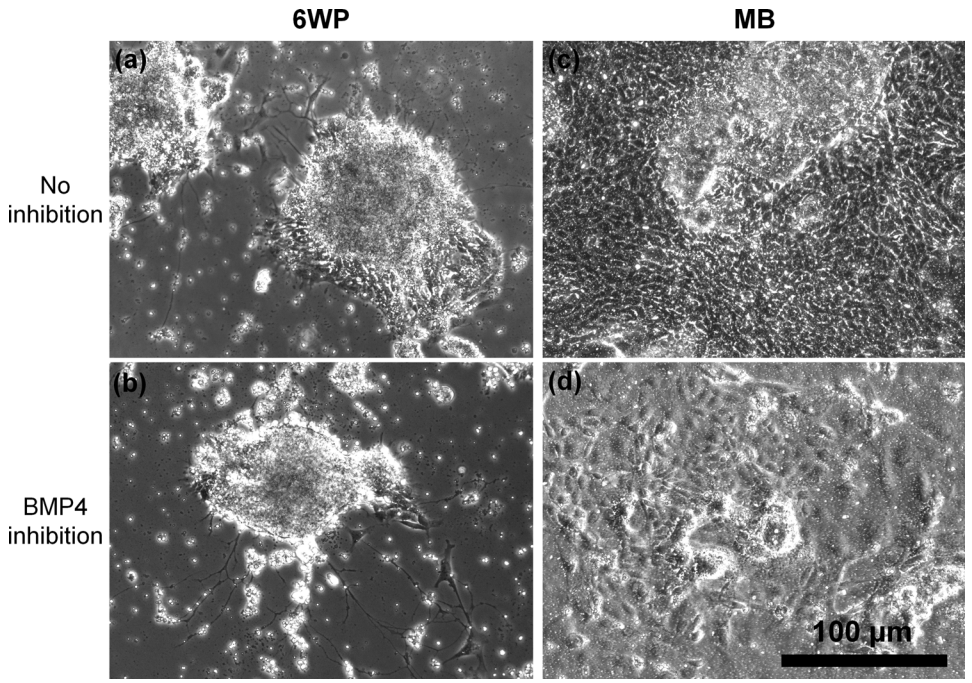

FIG. 6. Morphology of the differentiated cells in the 6WP ((a) and (b)) and MB ((c) and (d)) at day 8 without ((a) and (c)) and with the inhibition ((b) and (d)) of BMP4 by its antagonist Noggin. In the 6WP, there is no apparent change in the cell morphology, whereas clear morphological change can be observed in the MB by the inhibition. Scale bar represents 100 μm.

signaling.[24] Therefore, role of upregulated cell-secreted BMP4 on mESC pluripotency[11] and differentiation (in this study) in the MB correlated well with the respective role of exogenous BMP4 in the conventional macro-scale cultures.

In both pluripotency[11] and differentiation (in this study) cultures, microenvironment was dominated by BMP4 signaling in the MB whereas BMP4 signaling was negligible in the 6WP. mESCs and differentiated cells secret BMP4 moderately.[25] Owing to the small volume of the MB, moderately secreted BMP4 presumably reached a threshold level to activate BMP4 signaling thereby mESC behaviors in the MB. However, in the large volume of 6WP, moderately secreted BMP4 would not reach the threshold level to induce signaling activity. As a result, mESC culture or differentiation in the 6WP was prominently influenced by autocrine FGF signaling.

BMP4 was remarkably upregulated in the MB compared with the 6WP culture (Fig. 5(e)). This upregulation might be facilitated by two mechanisms: (1) activation of positive feedback mechanism by a relatively higher concentration of BMP4[25] in the small volume of MB and (2) BMP4 expression in differentiated mesodermal cells.[27,28] Although expressions of meso- and endo-dermal markers were downregulated by inhibition of BMP4 signaling in the MB (Fig. 7(b)), BMP4 expression did not change significantly (relative expression levels were 0.42 ± 0.22 and 0.28 ± 0.09 for non-inhibited and inhibited one, respectively). Therefore, activation of the feedback mechanism of BMP4 might have the prominent role for BMP4 upregulation in the MB. For clear distinction between the mechanisms, it is necessary to investigate differentiation at different time points, which is our future interest.

Although BMP4 was only upregulated in the MB among various soluble factors (Fig. 5(e)), accumulation of other factors in the small volume might have some influence. For example, besides BMP4, accumulation of Nodal and Activin (can induce meso- and endo-dermal differentiation in mESC)[24] in the small volume of the MB might have role in augmenting meso- and endo-dermal differentiation in the MB. However, inhibition of neuronal differentiation together with augmentation of meso- and endo-dermal differentiation and upregulation of BMP4 strongly

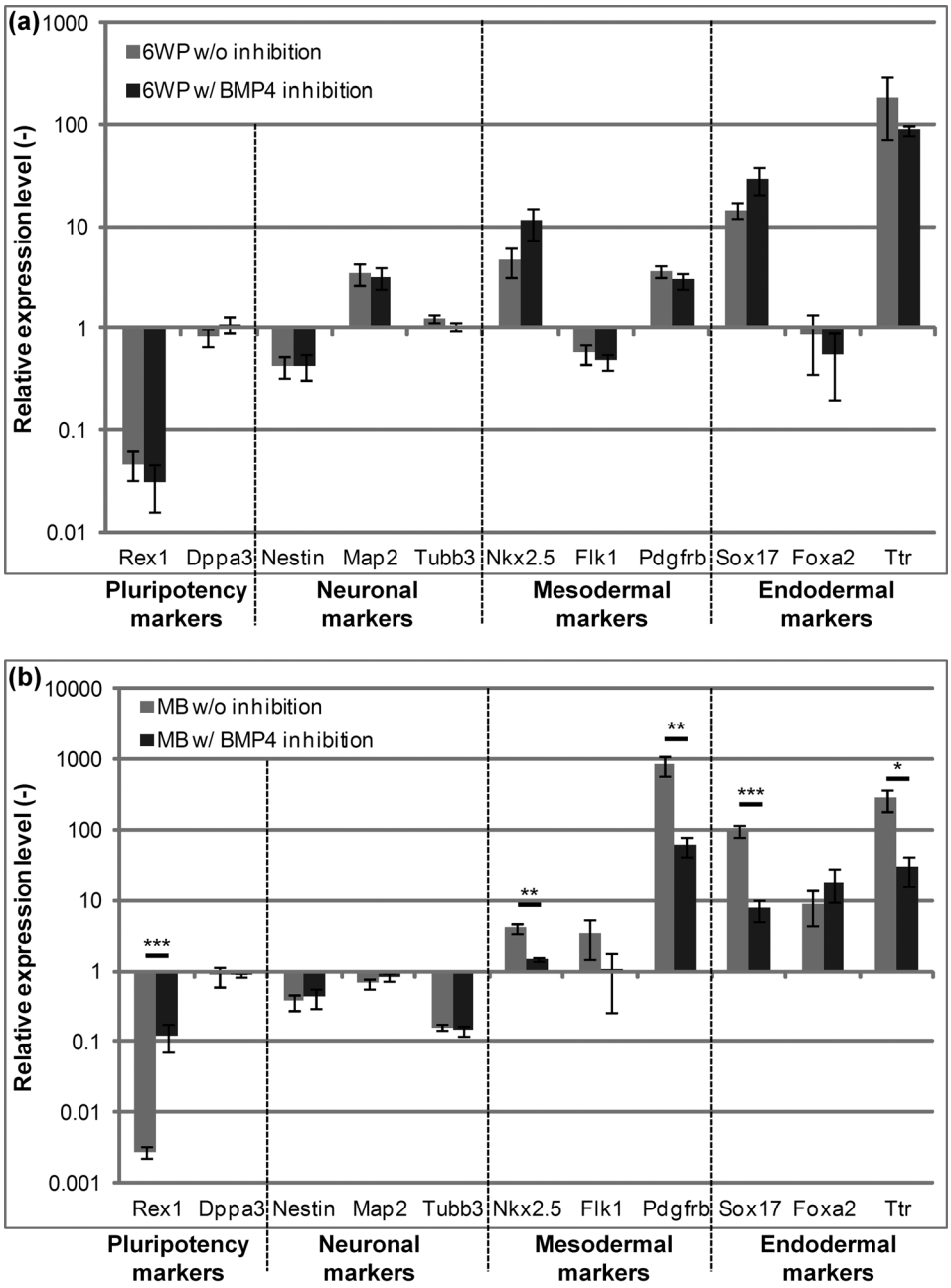

FIG. 7. Relative gene expression levels (in Log_{10}-scale) in the 6WP (a) and MB (b) cultures with and without the inhibition of BMP4 by its antagonist Noggin. Expression level one represents gene expression in the undifferentiated mESCs cultured for 2-days. Columns and error bars represent mean ± SEM of three to four cultures. Statistical significances are shown using symbols * ($P < 0.05$), ** ($P < 0.01$), and *** ($P < 0.001$).

suggested prominent role of BMP4 in the MB. Zhang and co-workers observed that addition of BMP4 in mESC differentiation culture by using knockout serum (KSR; the major constituent of CD-BM) containing medium resisted overall differentiation (i.e., small decrease of pluripotency markers) as well as inhibited neuronal differentiation.[26] Although cell-secreted BMP4 inhibited neuronal differentiation in the MB (Fig. 5(b)), it did not resisted overall differentiation of mESC culture (Fig. 5(a)). Therefore, signaling of various soluble factors presumably acted synergistically with BMP4 and formed an adequate signaling environment in the MB for inducing alternative fate choices of mESCs.

To assess endodermal differentiation, we analyzed expression of Sox17, Foxa2, and Ttr (Figs. 5 and 7) which are common markers for primitive endoderm and definitive endoderm.[20,24,29] Cell-secreted BMP4 dependent upregulation of these makers in the MB correlated well with their upregulation by exogenous BMP4.[24] In spontaneous differentiation of mESCs, mesoderm commitment occurs during first week whereas definitive endoderm occurs during second week of differentiation.[30] Therefore, endodermal differentiation in the MB as well as in the 6WP after 8 days of differentiation represented mostly primitive endoderm rather than definitive endoderm. Longer period differentiation of ESCs and EB in an enriched signaling environment to investigate emergence of definitive endoderm and developmental process, respectively, are potential applications of the MB.

IV. CONCLUSION

In this study, we differentiated mESCs in a diffusion dominant microenvironment formed in a membrane-based two-chambered MB to realize adequate signaling of cell-secreted factors by maintaining enough nutrient supply. Microenvironment formed in the MB facilitated meso- and endo-dermal differentiation rather than the default neuronal differentiation owing to enhanced signaling of cell-secreted BMP4. In contrast, in macro-scale culture (e.g., 6WP), cell-secreted BMP4 signaling was negligible and showed prominent neuronal differentiation. Therefore, the developed microbioreactor realized autoregulatory action of soluble factors which cannot be realized in macro-scale culture and provided an environment which enhanced ESC self-capabilities. This microbioreactor will be a useful tool to differentiate EB in an *in vivo* mimicking adequate soluble factor signaling environment for better understanding of developmental toxicology. Moreover, the understanding of this study will be useful to optimize autocrine/paracrine signaling environment in microbeads which are utilized in mass production of differentiated cells from ESCs.

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